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(54) Title: CYTOLYSIS INHIBITOR PROTEINS (CLI) AND DNA SEQUENCES CODING FOR SAID PROTEINS (57) Abstract A cytotoxicity inhibitor protein, DNA coding therefor, plasmids containing said DNA, host cells containing said plasmids and monoclonal antibodies to said protein are provided, as well as a method of isolating the cytotoxicity inhibitor from bodily fluid, a method of making the cytotoxicity inhibitor by recombinant technology, immunological methods of use of said antibodies and methods of treating various disease states.		

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CYTOLYSIS INHIBITOR PROTEINS (CLI) AND DNA SEQUENCES
CODING FOR SAID PROTEINS

Background of the Invention

5 The invention relates to a novel blood plasma
component having a strong inhibitory effect on the target
cell lysis mediated by terminal complement proteins, such
as, for example, by perforin secreted by killer cells or
by the α -toxic of Staphylococcus aureus. The component
10 likewise plays possibly an important role in lipid
metabolism.

 Two effector systems, complementing each other,
ensure immunological defense against entering viruses,
bacteria, virus-transformed and virus-infested cells,
15 endogenous cells which are altered in malignant fashion
or due to ageing, against sperms in the female genital
tract, and against exogenous material of the environment.

 The cellular effector system consists of specially
matured immune cells, the so-called cytotoxic T-lympho-
20 cytes and the natural killer cells. Lymphocytes and
natural killer cells are capable, with the aid of
inherent surface receptors, of recognizing exogenous
structures on other endogenous cells indicative of
abnormal states or in interstitial tissue, as well as
25 structures on foreign materials such as, e.g., invading
microorganisms, and of effectively preventing the
spreading and proliferation of these exogenous materials,

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endogenous cells and microorganism. Activated cytotoxic lymphocytes and natural killer cells enter into intimate contact with the target cells recognized as foreign and release membrane-damaging molecules in a controlled fashion, the lymphocytes and killer cells themselves being protected against these molecules. The actual membrane-damaging molecule of the killer cells is the so-called perforin possessing high affinity for the lipophilic membrane of the target cell. Several molecules of perforin penetrate into the double-layered membrane of the target cell and thereafter form a cylinder-like water-permeable channel transversely through the lipid double membrane of the target cell. Water molecules and electrolyte salts can freely move through the hydrophilic inner space of the transmembranous channel. Thereby the osmotic equilibrium of the cell is disturbed, and the target cell will die due to the influx of sodium chloride- and calcium-containing water from the extracellular environment

The humoral system of immune defense consists of a plurality of soluble plasma proteins which interact with one another and form a finely staggered cascade similar to that of the clotting system. The actual terminal step in complement activation leads to membrane damage to the target cell according to the same principle as in case of perforin-caused lysis of the target cells. Grouping together of the terminal components of the complement system is initiated by the cleavage of complement protein C5 into two fragments C5a and C5b. The carboxy-terminal main fragment C5b unites with C6, C7 and C8 to a macromolecular complex, the hydrophobicity of which increases considerably with the addition of C7 and C8 and which can bind to the lipid double membrane. The C5b-8 complex proper does not as yet form a functional pore in the lipid membrane. Membrane-bound, it takes over the

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part of a receptor controlling the addition of several C9 molecules to form a permeable membrane channel.

Soluble perforin monomers and the stable, soluble C5b-6 complex can move away from the site of their release and/or generation and can diffuse into the surroundings so that neighboring healthy cells could be affected. Therefore, it has been supposed for a long time that effective regulatory mechanisms exist which prevent uncontrolled propagation of potentially lytic molecules.

Summary of the Invention

The present invention provides purified natural inhibitor for the known immunological effector molecules, i.e., for perforin stemming from T cells and killer cells and for the terminal complement proteins of human plasma, as well as inhibitors produced by genetic engineering.

The invention further provides DNA which codes for the specific protein portion of a novel blood plasma component and to a purification process for the natural blood plasma component characterized by the CLI protein molecule and exerting an inhibitory effect on the target cell lysis mediated by complement proteins and on perforin isolated from killer cells. The invention especially concerns the nucleotide base sequence of the CLI-1 clone depicted in Figure 2 and the amino acid sequence derived therefrom. The invention furthermore provides the plasmid pGEM4/CLI-1 shown in Figure 1; host organisms transformed with this plasmid; and cytotoxic inhibitor from human plasma isolated for the first time.

This invention furthermore provides hybridoma cell lines, e.g., hybridoma cell line CLI-9, the monoclonal antibodies, e.g., monoclonal antibody CLI-9 produced by the hybridoma cell line CLI-9, and the use of said antibodies for the isolation and purification of CLI from

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human plasma and its use for the quantitative determination of the natural blood plasma component, of the polypeptide coded by CLI cDNA, and of the CLI-associated reaction products in biological fluids.

5 Finally, this invention also involves the pharmaceutical utilization of CLI for therapeutic and prophylactic purposes.

10 Upon further study of the specification and appended claims, further objects and advantages of this invention will become apparent to those skilled in the art.

15 Various other objects, features and attendant advantages of the present invention will be more fully appreciated as the same becomes better understood when considered in conjunction with the accompanying drawings, and wherein:

20 Figure 1 shows a scheme of the recombinant pGEM4/CLI-1 plasmid containing the entire coding sequence for the cytolysis inhibitor, and several important restriction scission sites. The plasmid has an ampicillin resistance gene coding for the enzyme beta-lactamase, and includes the Sp6 and, respectively, T7 promoter in the direct vicinity to the cloning scission site BamHI in the polylinker. The two ends of the cDNA insertion are provided with the adapter oligomers A and B
25 according to Haymerle, H., Herz, J., Bressan, G.M., Frank, R. and Stanley, K.K., Nucleic Acids Res. 154, 8615-8624 (1986). This plasmid was deposited in E. coli K12 on March 28, 1989 with the DSM (German Microorganism Depository) under DSM No. 5269;

30 Figure 2 shows the nucleotide base sequence of the CLI-1 clone and the amino acid sequence derived therefrom in the line therebelow. The amino-terminal sequences of the a-chain (amino acids 1-205) and the b-chain (206-427) are underlined. On the left-hand margin of the
35 figure, the numbering is indicated for the nucleotide

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bases, and on the right-hand margin of the figure, the numbering of the amino acids is set forth;

Figure 3 shows the results of the Southern analysis: the position of the molecular weight markers is indicated in kilobases on the left-hand margin of the figure;

Figure 4a shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (10% polyacrylamide proportion) under nonreducing (left) and reducing conditions (right);

Figure 4b shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (12% polyacrylamide proportion);

Figure 5 shows a Western blot identification (bars 1 to 3') of CLI in human plasma (1), in purified C5b-9 complex (2), in seminal fluid (3 and 3'). Bar 4 shows the proteins of the seminal fluid after staining the SDS gel with "Coomassie" blue;

Figure 6 shows the inhibition of complement lysis by CLI: CLI inhibits lysis of the erythrocytes by soluble C5b-6, C7, C8 and C9 in a concentration-dependent way (bottom curve), but does not inhibit lysis of C5b-7 erythrocyte intermediate stages by C8 and C9 (top curve);

Figure 7 shows the inhibition of perforin-mediated erythrocyte lysis at various CLI concentrations;

Figure 8 shows the inhibition of staphylococcus- α -hemolysin mediated erythrocyte lysis by CLI. Staphylococcus-A-hemolysin is preincubated with varying CLI concentrations at 37°C in GVB buffer and thereafter mixed with a rabbit erythrocyte suspension in GVB buffer. Subphysiological CLI concentrations (15 μ g/ml) almost completely suppress the toxin-caused lysis of the erythrocytes; and

Figure 9 shows that ApoA-I-CLI complexes appear in the electron microscope as spherical structures having a diameter of 9.0 ± 1 nm.

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The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

5 From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and
10 conditions.

 It was found during the characterization of the soluble terminal complement complex with the aid of various monoclonal antibodies (which were raised against said complex) that terminal complement complex contains,
15 in addition to the already known S-protein/vitronectin, another still unknown protein. This unknown protein was located by a monoclonal antibody, CLI-9, which, contrary to the expectation, did not recognize the S-protein. It was found that this protein, cytolysis inhibitor (CLI),
20 is not a component of the membrane-bound, lytic complement complex.

 By means of affinity chromatography with the aid of the monoclonal antibody CLI-9, according to routine methods, the corresponding component from human plasma
25 was isolated in a functionally active form and an amino acid sequence was derived therefrom using standard protein sequencing technology.

 The amino acid sequence of part of the glycoprotein CLI was determined by sequencing the first twenty amino-
30 terminal amino acids in accordance with the Edman degradation method. Synthetic DNA probes were then prepared with the aid of these sequences and used to

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screen a human liver-specific cDNA library, according to routine methods recited in Example 1. The novel gene was then cloned from the human genome. This gene codes for a protein molecule carrying the international name of
5 "Cytolysis Inhibitor" (CLI or ZLI). This polypeptide is an essential and characteristic part of a novel blood plasma component with the above-mentioned properties.

After isolating the recombinant plasmid pGEM4 of the clone CLI-1 according to a routine method, this plasmid
10 was analyzed with the aid of restriction enzymes. The size of the DNA insertion in the pGEM4 vector was determined.

Purified CLI was found to have a molecular weight of 70 kilodaltons under nonreducing conditions and a
15 molecular weight of 35 kilodaltons under reducing conditions in sodium dodecyl sulfate gel electrophoresis. The protein was also shown to be a glycoprotein. A comparison with all heretofore known sequences published in the literature showed that the complete protein
20 structure of CLI and the corresponding complete nucleotide base sequence had not been determined heretofore.

The term "CLI" as used herein refers to the glycosylated and unglycosylated form of the protein,
25 having the biological activity of inhibiting cytolysis mediated by cytolytic proteins, such as, e.g., complement or perforin, as well as to all isoforms of CLI which may differ from the natural glycosylated form found in human serum by their carbohydrate content. The term also
30 includes therefore the CLI of seminal plasma which may have other carbohydrate structures.

Based on the immunological and molecular-biological identification of the glycoprotein CLI, a novel component was isolated from human plasma in adequate amounts with
35 the aid of monoclonal antibodies, was purified, and was

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investigated for its inhibitory effect. The isolated blood plasma component counteracts undesired cellular destruction. It neutralizes, in a way dependent on concentration, the cytolytic potential of C5b-7 complement complexes in the extracellular fluid and in the plasma. The sensitivity of endogenous cells to complement and perforin lysis is diminished, and the threshold for cellular damage is increased. The glycoprotein CLI also occurs in human seminal fluid.

Intensive studies of the natural CLI blood plasma component (Example 2f) yielded the following results. In human blood plasma, CLI is fixedly associated with the apolipoprotein A-I (ApoA-I), which transports predominantly phospholipids and free as well as esterified cholesterol. In lipoprotein gel electrophoresis (Paragon system, Beckman), the ApoA-I-CLI particle migrates in the pre-beta zone. Since the ApoA-I concentrations in the plasma are about 20 times higher than the CLI concentrations, about 2.5% of the entire plasma ApoA-I is present in the form of an ApoA-I-CLI complex. High-density lipoproteins of the pre-beta fraction represent, on the average, a proportion of $4.2 \pm 1.8\%$ of the entire ApoA-I in the plasma. Therefore, the predominant proportion or even the entire fraction of pre-beta HDL could consist of ApoA-I-CLI particles. Thus, the term "natural CLI blood plasma component" as used herein refers to the entire apolipoprotein A-I (ApoA-I)/CLI/lipid complex found in whole human blood. It is further noted that this complex may be heterogeneous, e.g., it may bind additional components of lipid transport pathways, cholesterol-ester-transferin protein (CETP), lecithin cholesteryl acyltransferase (LCAT) and/or apolipoprotein A-II. The lipid content of the HDL-CLI complex can vary among

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individuals and even in the same individual after eating or during fasting.

In earlier works (Castro and Fielding, Biochemistry 27:25-29, 1988), it was demonstrated that cellular cholesterol is primarily taken up by pre-beta HDL and then is further transported to alpha HDL and LDL. Consequently, ApoA-I-CLI particles could represent an important link in the transfer of excess cellular cholesterol from the cell to LDL and HDL and in the cholesterol supply of peripheral tissues via LDL and HDL in the reverse direction. The CLI-HDL particles described by the inventors could be significant in total for the antiatherogenic effect of HDL.

The natural CLI blood plasma component isolated with the aid of the monoclonal antibody CLI-9 is capable, due to its special biochemical properties, of neutralizing the membrane-binding and membrane-inserting activity of the nascent complement complexes and of the monomeric perforin. The biological activity of the purified blood plasma component could be mediated by the polypeptide chain, the carbohydrate proportion associated therewith, or by CLI-associated lipid proportions.

The anticytolytic activity of CLI was confirmed by in vitro experiments with sheep erythrocytes. The efficiency of the erythrocyte lysis was hampered with increasing CLI concentrations. The effective CLI concentrations are in a range lying in the proximity of or somewhat below the natural plasma level.

The complete cDNA sequence of CLI was isolated from a liver-specific gene bank and analyzed with the use of methods of genetic engineering and based on the provided partial amino acid sequence. It is noted that this sequence coincided with an already published partial sequence.

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Southern blot analysis demonstrates that CLI is represented merely by a single gene in the genome of man and in rats. The existence of very similar or highly homologous genes, i.e., the presence of a gene family, was thereby excluded with high probability. This result is particularly important in view of the fact that it was possible, with the aid of the monoclonal antibody CLI-9, to unequivocally identify the cytotoxic inhibitor also in seminal fluid. The molecular weight as well as the reactivity with the monoclonal antibody CLI-9 support the conclusion that the protein in human seminal fluid involves the same gene product.

The clone CLI-1 which was isolated as described herein has a length of 1651 base pairs and exhibits an open reading frame starting with the 199th nucleotide base. The amino acid sequence derived from the nucleotide base sequence has 448 amino acid residues. The first 21 amino acids form a typical hydrophobic signal peptide which is removed during translocation into the rough endoplasmic reticulum. The mature secreted protein thus begins with the sequence D-Q-T-V-S-D-N-E (aspartic acid, D, is number 1). The double-chain form of CLI is due to the fact that under reducing conditions, even prior to secretion of CLI into the human plasma, the peptide bond between arginine-205 and serine-206 is hydrolyzed by a still unknown cellular protease.

The structural properties of CLI are typical of apolipoproteins. The predicted α -helix content of CLI is 41%. There are several helical segments of amphiphilic nature (residues 1-76, 150-170, 215-240, 300-350 and 406-420) which are conserved across human, rat and quail species. Similar amphiphilic structures are shared by all apolipoproteins, e.g., ApoA-I and A-II. These predicted secondary structural elements are likely to interact with lipid bilayers and the lipids of the CLI-

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HDL complex. The hydrophobic surface of these protein segments are expected to mediate contact with the hydrophobic leaflet of lipid bilayers, the lipid micelles of CLI-HDL complexes and the hydrophobic, membrane-
5 inserting domains of channel-forming, cytolytic proteins of the terminal complement system, the killer cell-derived lytic protein perforin and cytolytic proteins of pathogenic microorganisms. The hydrophilic surfaces are exposed to the surrounding hydrophilic fluid (plasma,
10 interstitial fluid) and determine the water solubility of the CLI-HDL complexes.

By "corresponding to" is meant sequences that are identical to the DNA and amino acid sequences particularly identified herein or are different due to
15 natural variations which produce proteins having a biological activity of CLI. Thus, in addition to the particular DNA sequence indicated herein, this invention also encompasses DNA sequences coding for the same protein but differing due to degeneracy of the DNA code,
20 as well as sequences coding for variants, e.g., allelic variants, of the CLI protein which have the biological activity of CLI, i.e., of inhibiting cytolysis mediated by cytolytic proteins.

This invention also encompasses DNA sequences coding
25 for synthetic mutant CLI proteins (muteins) having a biological activity of CLI. Thus, in addition to the particular amino acid sequence indicated herein, this invention also encompasses synthetic variants of the CLI protein which have the biological activity of inhibiting
30 cytolysis mediated by cytolytic proteins, as well as such variants having such activity or which are capable of binding to antibodies that bind specifically to CLI. Suitable variants can be recognized, identified and made
by the skilled worker according to the particular use
35 required by reference to the physical characteristics of

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the natural CLI protein, as outlined above, including, e.g., the helical conserved regions. Thus, for example, by replacing moderately hydrophobic residues with more hydrophobic amino acid residues, the stability and lipid affinity of the CLI-HDL complex can be increased; conversely, by introducing highly hydrophilic residues and generating additional asparagine-linked glycosylation sites (Asn-X-Ser/Thr) at the water interface, the water solubility and half-life of CLI and CLI-HDL complexes could be increased.

The DNA sequences, plasmids, cytolytic proteins, antibodies and cell lines of this invention can be produced by any and all means conventional in the art, based upon the disclosure of this invention. Specific and general methods for making these products are described above and below.

Natural CLI can be purified from human bodily fluids by any means routine to one of ordinary skill in the art, given the disclosure of this invention. In a preferred method, the monoclonal antibodies to CLI of this invention are utilized for this purpose by attaching them to an activated solid support material, e.g., Sepharose, and contacting a solution containing the bodily fluid, e.g., plasma or seminal fluid, with the antibody-solid support matrix. The CLI will bind to the antibodies, while the rest of the constituents of the bodily fluid do not. The CLI is then obtained in highly purified form upon release from the antibodies, e.g., by use of a glycine/NaCl buffer.

CLI can also be produced by recombinant DNA technology according to methods well-known to those of ordinary skill in the art. Given the amino acid sequence disclosed herein, as well as the DNA sequence also disclosed, probes for the CLI gene can be routinely synthesized which are complementary to DNA sequences

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expected to be found in a CLI gene. By using such probes, DNA sequences for the gene encoding the CLI gene, e.g., sequences contained in the cloning plasmid pGEM4/CLI-1, can be isolated from, e.g., endogenous
5 plasmid sequences in genomic libraries, and cloned into an expression vector suitable for the expression of the gene, according to techniques which are well-known to one of ordinary skill in the art.

For uses in which glycosylation of the final protein
10 is not required, the DNA sequence can be expressed in either non-glycosylating procaryotic or eucaryotic organisms. For uses in which glycosylation is required, the DNA sequence may be expressed in organisms, e.g., eucaryotic cells, which endogenously perform the required
15 glycosylation. Alternatively, non-glycosylated protein may be glycosylated in vitro according to well-known methods, e.g., by use of the appropriate glycosylating enzymes.

The protein can then be isolated and purified from
20 the cells by means appropriate to the cell type in which it is expressed; i.e., if produced in a cell type which secretes the protein, it may be purified from the cell-free medium separated from the expressing cells. If the protein remains intracellular, then it can be isolated by
25 any one of numerous means known in the art, e.g., the cells are mechanically or osmotically ruptured, insoluble cellular debris is removed from the soluble fraction, and the desired protein is purified by chromatographic means, ionic precipitation, HPLC, etc., including the affinity
30 chromatography method also described herein using an antibody specific for the CLI protein bound to a solid support.

Antibodies specific for CLI are made by methods well known to one of ordinary skill in the art, e.g., by
35 multiply injecting purified CLI into mice or rabbits and

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purifying the thus-elicited antibodies from the animals' serum, e.g., by affinity chromatography using CLI affixed to a support medium. Monoclonal antibodies are also made by well-known methods, e.g., according to the well-known method of Kohler and Milstein.

Having antibodies specific to the desired protein in hand, development of immunoassay tests for the detection and quantitation of CLI, CLI-fragments, and CLI-containing complexes, e.g., CLI-ApoA-I and/or CLI-HDL complexes, is a routine matter for one of ordinary skill in the art. Test development protocols are well-known and can be modified by routine experimentation to customize the assay for the detection of CLI. Such immunoassay tests can be used to detect and establish levels of CLI present in any normal and disease state of interest in animals, including humans, according to methods well known to one of ordinary skill in the art. Thus, for example, serum can then be tested for the presence of normal/abnormal levels of CLI, either alone or as a complex with various other materials, e.g., with ApoA-I, or with HDL. Also, seminal fluid can be routinely tested for the presence of normal/abnormal levels of CLI. These tests can be routinely performed and quantitated, e.g., according to well-known methods. Similarly, antibodies according to this invention can be used to perform immunohistological differential diagnoses and in vivo diagnoses, e.g., according to the usual methods known in the art. In particular, for the in vivo diagnoses, an antibody of this invention can be labelled in a way detectable by external detecting means, e.g., radiolabelled for X-ray imaging or labelled with a paramagnetic species for magnetic resonance imaging, the labelled antibody administered to the patient, and the imaging procedure performed.

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CLI-containing complexes, e.g., CLI-ApoA-I and CLI-HDL, can be isolated from bodily fluids using routine methods known to one of ordinary skill in the art. For example, they can be isolated electrophoretically, as described above, or the anti-CLI-antibody/support material affinity chromatography method can be employed, with or without concurrent use of anti-ApoA-I antibodies. Furthermore, reconstituted complexes comprising isolated CLI, either purified natural isolated CLI or CLI produced by recombinant technology, as described above, can be routinely made in the presence or absence of lipids, e.g., phospholipids, and optionally also in the presence of cholesterol, e.g., by mixing the components in the presences of detergents to totally solubilize the proteins and lipids (Ann. Rev. Biophys. Chem. 15, 403-456 (1986)). The detergent is then removed either by dialysis or molecular sieve chromatography over a gel filtration column that includes the detergent and excludes the final reconstituted product. Suitable detergents include, e.g., sodium cholate, sodium taurocholate or octylglucoside. Egg phosphatidylcholine and free cholesterol are commercially available, and lipid-free ApoA-I can be isolated from human plasma by the method of Peitsch et al. *Analyt. Biochem.* 178, 301-305 (1989).

The cytolytic inhibitor protein of this invention can be used as a therapy for conditions which are mediated by cytolytic protein-mediated cell lysis. Thus, increasing the CLI plasma level, e.g., by administration of exogenous CLI, represents a therapeutic approach for counteracting increased systemic complement activity in the body caused by various disease and traumatic states, as well as the activity of perforin-induced cell lysis, and/or activity of other membrane-active cytolytic proteins.

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Further approaches made possible by this invention include control of plasma levels of the cytolysis inhibitor by pharmaceutical or biologically active compounds altering the gene expression of the cytolysis inhibitor in the liver or in other tissues, e.g., the Sertoli's cells. Immunological methods for determining the CLI concentrations in human body fluids are also encompassing this invention.

CLI has been postulated to have a role in human fertility, in that it is involved in preventing premature lysis of the cell membrane of the sperm prior to fertilization of the egg. Therefore, congenital or acquired lack of CLI may be a cause of male infertility. Similarly, the presence of CLI-specific antibodies in intravaginal and/or intrauterine fluid may also be a cause of infertility. Therefore, CLI or fragments thereof, including the carbohydrate portion of the glycoprotein, are used to develop a vaccine against CLI, thereby producing in a male immunized therewith a contraceptive effect. Such a vaccine is produced and administered according to well-known methods.

The invention additionally includes the systemic or local administration of CLI for the treatment of tissue-destroying pathological conditions evoked by complement and killer cells, and the local or systemic administration of CLI for detoxification of membrane-active peptides and cytolytic proteins secreted by pathogenic organisms, for example by bacteria (inter alia Staphylococcus aureus, Escherichia coli), fungi, or insects (for example, in the poison of honeybees, wasps, hornets, bumblebees).

The CLI-protein, glycoprotein, protein fragments and/or complexes, e.g., CLI-ApoA-I or CLI-HDL, are administered for the various treatment protocols analogously to other inhibitory proteins, e.g., protease

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inhibitor, which are used in animal, especially human, medicine. Particular dosages for a given indication, patient and compound or complex can be routinely determined, e.g., by using standard pharmaceutical procedures well known to practitioners.

Furthermore, due to the nature of the cytolytic process, and the intimate involvement of CLI in controlling it, the presence of CLI in particular locations in tissues throughout the body (e.g., in above-background amounts) is an indication of various types of cellular damage and conditions. Thus, for example, CLI can be involved in tissue damage resulting from, e.g., chemical, metabolic, hormonal, ischemic, mechanical, and immunological causes, as well as in response to involution of normal human tissues. Furthermore, CLI localization during cytolytic tumor therapy is useful to detect the progress and/or regression of such tumors. Thus, the antibodies to CLI have a particular utility for the immunological detection, quantitation, immunohistological differential diagnosis, and localization of CLI and/or CLI-containing complexes in patients, including in bodily fluids, tissue samples, histological specimens, and, in the case of in vivo imaging as discussed above, in a whole patient, by use of appropriately labelled antibodies, which can be labelled according to any of the many suitable ways known to one of ordinary skill in the art for labelling proteins in general and immunological molecules in particular.

For all applications noted herein, CLI and variants thereof and muteins, as well as the DNA sequences, plasmids and antibodies coding therefor or binding thereto, are similarly useful. Furthermore, muteins, which may or may not have a particular biological activity of CLI, can be used to map the binding sites of monoclonal antibodies and to study the domain interaction

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between ApoA-I and CLI, and for quality control, e.g., of production processes.

5 Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

10 In the foregoing and in the following examples, all temperatures are set forth uncorrected in degrees Celsius and unless otherwise indicated, all parts and percentages are by weight.

15 The entire disclosures of all applications, patents and publications, if any, cited above and below, and of corresponding application German P 3933850.9, are hereby incorporated by reference.

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ExamplesExample 1Isolation of the Cytolysis Inhibitor (CLI) Gene

5 (a) Designing of a Probe and Production
of Synthetic Oligonucleotides

Nucleotide acid sequences are derived via the genetic code from the partial amino acid sequence of the two chains of CLI, and the corresponding oligonucleotides are synthetized as hybridization probes. Since the

10 genetic code is degenerated, i.e. in most cases several codons can code for the same amino acid, two long oligonucleotides are synthetized and, in accordance with the rules by Lathe, J. Mol. Biol. 183 : 1-12, 1985, only those codons are selected which, based on statistic-

15 al empirical values, can be found with maximum probability in human genese for the respective amino acids. The partial sequence DNEIQEMSNQG is selected for probe 1, and the partial sequence PYEPLNFHAMFQPFLEM is chosen for probe 2. These protein sequences are determined in

20 accordance with Example 2 and/or according to Murphy et al., J. Clin, Invest. 81 : 18589-1864, 1988. The resultant synthetic hybridization probes have the following nucleotide base sequences for probe 1: 5'-GAC AAT GAG CTG CAG GAG ATG TCC AAC CAG GG-3'; for probe 2:

25 5'-CCC TAT GAG CCC CTG AAC TTC CAC GCC ATG TTC CAG CCC TTC CTG GAG ATG-3'. Comparison with the subsequently determined, correct cDNA sequence shows that both probes deviate merely by three nucleotide bases from the correct cDNA sequence, and probe 1, a 32-oligomer, codes for

30 the amino acids 6-15 of the a-chain and the probe 2, a 51-oligomer, codes for the amino acids 7-23 on the b-chain.

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(b) Terminal Marking of the Oligomer Probes

The probes are labeled radioactively on the 5'-terminus with the use of [γ - ^{32}P]dATP (Amersham) and T4 polynucleotide kinase (Pharmacia-LKB, Sweden).

5 The 20 μl reaction solution contains 70 mmol of tris-hydrochloride, pH 7.6, 10 mmol of MgCl_2 and 5 mmol of DTT, 3 pmol of the respective oligomer, 9 pmol of [γ - ^{32}P]dATP, and 6 units of T4 DNA polynucleotide kinase, and is incubated for 30 minutes at 37° C. The

10 free, nonused [γ - ^{32}P]dATP is separated from the oligonucleotides by gel filtration (NAP 25, Pharmacia-LKB, Sweden).

(c) Screening of a cDNA Library of the Liver

The so-called colony hybridization for bacterial

15 clones is performed with the use of large colony densities. Approximately 200,000 bacteria of a liver-specific cDNA library (Haefliger, J.-A., Jenne, D., Stanley, K.K., and Tschopp, J.: Biochem. Biophys. Res. Commun. 149 : 750-754, 1987) are spread onto 20

20 ampicillin-containing (50 $\mu\text{g}/\text{ml}$) agar plates having a size of 22 cm x 22 cm. The agar medium employed is 10 g/l of "Bacto-Trypton", 5 g/l of NaCl and 1.6% (wt./vol.) "BactoAgar". After cultivating the bacteria for 20 hours overnight at 37° C, the roundish colonies of a size of

25 about 1 mm are transferred by simple kiss method from the agar plates to nitrocellulose filters (Schleicher und Schüll). The bacterial colonies are further cultivated on the original agar substrates for 7 hours and in this way a replicate capable of growth is

30 provided for each colony on the nitrocellulose filters.

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The bacterial colonies on the nitrocellulose filters are solubilized by means of sodium dodecyl sulfate and sodium hydroxide solution. During incubation of the nitrocellulose filters in an alkaline solution, the bacterial DNA is released, denatured, and bound as single-strand DNA to the filter membrane. After several washing steps with neutral buffers, the DNA is firmly fixed to the nitrocellulose by heating (Davies, L.G., Dibner, M.D., and Battey, J.F., Basic Methods in Molecular Biology, Elsevier, New York, 1986). In order to prevent unspecific binding of the probe to the nitrocellulose, the filters are pre-hybridized for four hours at 50° C. The solution consists of 6 x SSC (1 x SSC contains 0.15 moles NaCl, 0.015 moles sodium citrate), 5 x Denhardt's solution (according to Maniatis, T., et al. in Molecular Cloning, Cold Spring Harbor Laboratory, New York, 1982), 0.1% sodium dodecyl sulfate, 10 mmol EDTA and 50 µg/ml salmon sperm DNA (= hybridization solution) subjected to ultrasound and denatured by boiling for five minutes. Hybridization with the 51 oligomer probe takes place at 50° C in fresh hybridization solution over a period of four hours. The concentration of the radioactive probe in the hybridization solution is 5×10^4 cpm/ml. The nitrocellulose filters are washed at 45° C in 1 x SSC and 0.1% SDS (weight/volume) for 60 minutes and exposed overnight to an X-ray film at -70° C. On the next day, the 51 probe is removed by boiling for 10 minutes in a 10 mmol EDTA and 1% aqueous glycerol solution. The filters are subjected to a second screening procedure with the 32 probe. A strong unequivocal signal with both probes is observed only on three places on the 20 filters. Bacterial clones from these sites are

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examined a second time by streaking and renewed cultivation on small agar plates. During this process, the bacterial density is reduced to such an extent that the colonies stand individually and are not intermingled with neighboring colonies. The analysis of respectively 30 colonies from the three positive regions finally results in 3 independent bacterial clones reacting with both probes.

10 (d) Characterization and Isolation of the
cDNA of the CLI-1 Clone

The recombinant plasmid of the CLI-1 clone is isolated according to a routine method and analyzed by restriction enzymes (L.G. Davies et al., Basic Methods in Molecular Biology, Elsevier, New York, 1986).
15 The DNA is cleaved with the restriction enzymes BamHI and KpnI. The resultant DNA fragments are separated by agarose gel electrophoresis, and the size of the DNA insertion in the pGEM4 vector is determined. The length of the cDNA amounts to 1.7 kb; there are no
20 internal BamHI and KpnI scission sites (Figure 1). Approximately 20 µg of the cDNA is separated from the pGEM4 vector plasmid by agarose gel electrophoresis and purified for DNA sequence determination.

(e) cDNA Base Sequence Determination

25 The cDNA of the CLI-1 clone is separated, after spontaneous ligation, by ultrasonic treatment in an ultrasonic water bath into randomly distributed subfragments and fractionated according to size by electrophoresis in 1.3% strength NA-agarose (Pharmacia).
30 Those fragments which fall within the molecular weight

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range from 300 to 600 bp are isolated, and the ends are repaired with the aid of T4 DNA polymerase so that smooth termini are produced. The cDNA fragments produced in this way are inserted into the SmaI restriction scission site of M13mp8 vector with the use of T4 DNA ligase. The single-stranded phages of the M13 vector are prepared according to standard methods (L.G. Davies et al., Basic Methods in Molecular Biology, Elsevier, New York, 1986), and the nucleotide sequence of about 50 subfragments is determined with the aid of the "Sequenase" kit (United States Biochemical Corporation). The nucleotide base sequence of the CLI-1 cDNA is determined at least once on both strands. The overlapping partial sequences are compared with the aid of a microcomputer and composed into the total sequence (Figure 2).

(f) Southern Analysis of the Genome of
Rat and Man

5 µg of whole DNA of man and of rat (Genofit, Geneva) are separated into fragments with the aid of the restriction enzymes BamHI, BglII, EcoRI and HindIII and then segregated electrophoretically in 0.8% agarose. After transferring onto a nylon mesh membrane by vacuum blot method (Pharmacia-LKB), the filter membrane is analyzed with the aid of radioactively labeled single-strand cDNA of the CLI-1 clone. The cDNA probe hybridizes with one to at most three fragments in the rat and human genome depending on which of the five restriction enzymes is utilized (Figure 3). The fact that it is possible to localize the complete cDNA sequence of CLI on a single restriction fragment in case of the rat (EcoRI) as well

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as in man (BamHI), proves that there is only a single gene for the biosynthesis of the cytolysis inhibitor in rat and in man.

Example 2

5 Purification, Biochemical Characterization and Identification of CLI with the Aid of Monoclonal Antibodies

=====

(a) Isolation of the CLI and S-Protein from Complement-Activated Serum

10 The alternative route of complement cascade is activated in 1 liter of human serum by the addition of insulin (10 mg/ml, Merck). The thus-produced soluble terminal complement complex, the so-called "SC5b-9" complex, is isolated according to Bhakdi and
15 Roth (J. Immunol., 127 : 576-582, 1981). In case of absence of target cell membranes during complement activation, this complex is produced by the uniting of the complement proteins C5b, C6, C7, C8, C9, S-protein, and CLI. In the presence of anionic detergents,
20 S-protein and CLI will dissociate from the complex. 250 mmol of deoxycholate (DOC) in the solid form is added to a solution of 0.6 mg to 1.2 mg/ml of SC5b-9 complex. This solution is incubated at 37° C for one hour in the presence of 2 mmol of PMSF. Respectively
25 4 ml is loaded onto a linear sucrose gradient (40 ml total volume, 10% by weight to 40% by weight of sucrose in 6.25 mmol of DOC, 10 mmol of tris-hydrochloride, pH 8.1, 50 mmol of NaCl and 7.5 mmol of NaN₃), and centrifuged for 3 hours at 4° C and 250,000 g's in a

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Beckman vertical rotor (model Vto-50). 2 ml fractions are collected from the bottom of the centrifuge tube. The fractions containing S-protein and CLI are pooled and concentrated approximately fivefold (Amicon PM10
5 membranes). The proteins are then separated with the aid of a gel filtration column ("Sephacryl" S-300, 1 cm x 60 cm column, Pharmacia). The equilibration buffer is 10 mmol of tris-hydrochloride, 50 mmol of NaCl, pH 8.1. CLI and S-protein elute in a peak.
10 The fractions are concentrated (protein concentration between 0.4 mg/ml and 0.6 mg/ml) and stored at -20° C.

(b) Preparation of Monoclonal Antibodies

500 µl of the mixture of S-protein and CLI are injected subcutaneously into female Balb/c mice in
15 complete Freund's adjuvant (1:1; vol./vol.). After 5 weeks, the injection of 500 µl of the antigen is repeated, this time in incomplete Freund's adjuvant. After another 5 weeks, 500 µl of antigen without adjuvant is administered intraperitoneally, and the
20 mice are then sacrificed after 3-4 days.

Myeloma cells are cultivated in RPMI 1640 medium containing additionally 10% inactivated fetal calf serum, 1% glutamine, 5,000 U/ml of penicillin, 5 µg/ml of streptomycin, and 0.02 mmol of 2-mercapto-
25 ethanol (myeloma medium). The myeloma cells are cultivated at 37° C in 12 Petri dishes having a diameter of 9 cm until they begin to become confluent. The myeloma cells and the cells from the spleen of an immunized mouse are mixed with each other in a
30 serum-free medium and pelletized at 1,200 rpm. The cell sediment is gently resuspended in 2 ml of

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the fusion solution (50% PEG 4000 in RPMI 1640) and mixed for 2 minutes at 37° C gradually by means of a pipette. The fusion solution is then diluted with 20 ml of RPMI 1640. Thereafter the cells are sedimented and distributed in 20 ml of fresh myeloma medium on 4 microtiter plates (96 chambers). The culture chambers contain macrophages obtained by flushing the peritoneal cavity of 2 mice. On each subsequent second day, the myeloma medium which, for the purpose of selection, then contains additionally 0.1×10^{-2} mmol of hypoxanthine, 4×10^{-4} mmol of aminopterin, and 1.6×10^{-2} mmol of thymidine (-HAT medium), is changed. Hybridoma culture supernatants are examined with the aid of an ELISA test for CLI-and S-protein-specific monoclonal antibodies. Positive hybridoma cells are cloned 3x and subsequently injected into Balb/c mice pretreated with 500 µl of pristane. Ascites fluid is removed by puncture from the abdominal cavity 2-3 weeks later (Jenne, D., Hugo, F., and Bhakdi, S., Biosci. Rep. 5 : 343-352, 1985). The monoclonal antibody, CLI-9, is not directed toward the S-protein and is utilized for affinity purification of CLI (Bhakdi, S., Jenne, D., Hugo, F., J. Immunol. Meth. 80 : 25-32, 1985). By immunization with purified CLI, further immunoglobulin-secreting hybridoma lines are established. The hybridoma line with the designation of CLI-9 was deposited with the European Collection of Animal Cell Cultures, Salisbury, SP4 0JG, Great Britain, under "provisional accession number" 89 052 602. The monoclonal antibodies secreted by CLI-9 react with reduced and unreduced CLI in the Western Blot analogously as in Example 2(e).

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(c) Affinity Purification of CLI

The monoclonal antibody CLI-9 is purified from 1 ml of ascites fluid. For this purpose, the ascites fluid is passed over a 2 ml protein A "Sepharose" column equilibrated in 10 mmol of tris-hydrochloride, pH 7.4, 150 mmol NaCl (subsequently called TBS). The antibodies are eluted with 10 ml of 0.2-molar glycine, pH 2.8, 0.5-molar NaCl. 4 mg of the purified antibody is subsequently coupled to 1.5 ml of cyanogen bromide-activated "Sepharose" (in accordance with the directions by the manufacturer, Pharmacia). For a second column, 20 mg of human immunoglobulin ("Sandoglobin", Red Cross, Berne) is coupled to 4 ml of cyanogen bromide-activated "Sepharose". 20 ml of fresh human EDTA plasma (10 mmol EDTA) is diluted with 10 ml of TBS and first applied to the human IgG affinity column (equilibrated in TBS). The unabsorbed proteins are thereafter passed through the anti-CLI affinity column. This column is washed with 10 ml of TBS buffer to which 1.5-moles NaCl has been admixed. Pure CLI is eluted with 10 ml of 0.2-moles glycine buffer, 0.5 moles NaCl, pH 2.8 (Figure 4). Approximately 0.5 mg of CLI can thus be obtained in a buffer volume of 5 ml. Purified CLI is dialyzed against TBS (16 hours) and either stored at 4° C for several days or deep-frozen (-20° C) in small portions (100 µl).

(d) Amino Acid Sequence of CLI

10 µg of the purified CLI are dialyzed against 10 mmol ammonium acetate, pH 8.0, and applied dropwise to a glass fiber membrane treated with "Polybrene". The individual amino acids are degraded stepwise in accordance with the method by Edman and analyzed with the aid of an HPLC unit (Applied Biosystems).

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(e) Detection of CLI in Plasma and in
Seminal Fluid

The so-called "Western Blot" analysis is especially well suited for identification of CLI. In this process,
5 1 µl of the 1:10 diluted seminal fluid or 10 µl of a 1:10
diluted serum solution is separated by SDS-polyacrylamide
gel electrophoresis (Laemmli, Nature 227 : 680-685,
1970) according to molecular weights. The proteins
are subsequently transferred by electrophoresis to
10 nitrocellulose membranes (Schleicher and Schüll,
15 cm x 10 cm) (Towbin, H., Staehelin, T., and Gordon, J.,
Proc. Natl. Acad. Sci. USA 76 : 4350-4355, 1979). The
electrophoresis buffer consists of 25 mmol tris-
hydrochloride, 192 mmol glycine and 20 vol-% methanol.
15 The proteins are transferred for 3 hours at 60 V and
220 mA. The nitrocellulose membranes are incubated for
one hour in saturation buffer (1% gelatin [Merck], 0.1%
bovine albumin [Böhringer], 20 mmol tris-hydrochloride,
pH 8.4, 150 mmol NaCl, 5 mmol EDTA and 0.02% sodium
20 azide), washed three times with TBS, and incubated
overnight with ascites fluid of the monoclonal antibody
CLI-9 (1:1000 dilution) in incubating buffer (= saturation
buffer without bovine albumin). After washing three
times in TBS (respectively 5 minutes), either a protein
25 A peroxidase conjugate (dilution 1:1000, Sigma) or an
anti-mouse IgG peroxidase conjugate (dilution 1:1000,
Dakopatis) is added thereto. The nitrocellulose mem-
branes are incubated for another two hours. The
peroxidase activity is determined with 4-chloro-1-
30 naphthol (Merck, 0.075%) in the presence of 0.01%
(vol./vol.) of H₂O₂ in a buffer mixture consisting of
20 vol-% methanol and 80 vol-% 10 mmol aqueous tris-HCl,

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pH 6.8. CLI can be clearly identified as a band in the molecular weight range of 70,000 daltons. The antibody CLI-9 reacts with unreduced human CLI, but not with reduced CLI or with CLI from other animal species.

5 (f) Biochemical Characterization of the
Blood Plasma Component Obtained by
Affinity Chromatography with the
CLI-9 Antibody

10 When examining the various elution fractions
of the anti-CLI affinity column (Example 2c) with the
aid of SDS polyacrylamide gel electrophoresis (SDS-PAGE),
with a high polyacrylamide proportion (Figure 4b), then
it can be seen that a further protein having a molecular
weight of 28 kilodaltons is regularly isolated with CLI as
15 a constant component. After SDS polyacrylamide gel
electrophoresis under nonreducing conditions, the 28
kilodalton component was blotted onto an "Immobilon"
membrane and added to the sequence on the amino-
terminal end in accordance with the method by Edman
20 (see Example 2d). The first ten amino acids of the
28 kilodalton component are Asp-Glu-Pro-Pro-Gln-Ser-Pro-
Trp-Asp-Arg and exactly coincide with the amino-terminal
sequence of the apolipoprotein A-I (ApoA-I). The
ApoA-I of the CLI complex shows the same mobility in
25 the SDS-PAGE as ApoA-I of purified HDL and reacts
with commercially available ApoA-I-specific antisera
(Behringwerke, Marburg). From this, the inventors
have drawn the conclusion that the 28 kilodalton com-
ponent represents the mature form of the intact ApoA-I.

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Since the same molecular weight form of ApoA-I is considered to be the specific protein marker of the high-density lipoproteins (HDL), the supposition presented itself that the ApoA-I-CLI complex could involve a new special HDL subfraction. Therefore, the inventors analyzed the lipid content and the lipid composition of the ApoA-I-CLI complex. The ApoA-I-CLI particle consists of 78% by weight of protein and 22% by weight of lipid. The lipids were separated by thin-layer chromatography into individual lipid fractions and quantified by comparison with commercially available lipid standards in situ on the thin-layer plate. The lipid proportion in mol-% is broken down as follows:

- 54% total cholesterol (unesterified to esterified cholesterol, 0.58)
- 42% phospholipids (85% phosphatidylcholine, 9% sphingomyelin, 6% phosphatidylethanolamine, and other phospholipids)
- 4% triglycerides.

Thus, the significant characteristic of the ApoA-I-CLI lipid particle is the very high protein proportion and the proportion of unesterified cholesterol which is high in comparison with esterified cholesterol. Based on the low lipid proportion of 22%, the ApoA-I-CLI lipid particle is not found in the classical potassium bromide density fraction of HDL ($d = 1.063$, 1.21 g/ml).

A further indication that the purified ApoA-I-CLI complex involves an HDL particle is constituted by the results of investigations with the electron microscope. ApoA-I-CLI complexes are presented in the electron microscope as spherical, homogeneous-appearing structures of uniform size (Figure 9). The average particle diameter amounts to $9.0 \pm 1.0 \text{ nm}$ and thus is similar to the

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HDL particles of size category 3 (8.5 nm to 9.6 nm), the smallest of the three size categories.

Example 3

Biological Activity of CLI

5 (a) Inhibition of Complement-Mediated Lysis by CLI

The functional, inhibiting properties of the natural CLI plasma component, as purified from plasma in accordance with Example 2(c) will be examined below

10 in the complement lysis test. For the lysis test, sheep erythrocytes (Biomerieux, 1×10^9 cells per ml) are utilized. The complementary proteins C5b-6 (Podack, E.R., and Müller-Eberhard, H.-J., J. Immunol. 124 : 332-336, 1980), C7 (Podack, E.R., Kolb, W.P.,

15 Esser, A.F., and Müller-Eberhard, H.-J., J. Immunol. 123 : 1071-1077, 1979), C8 (Kolb, W.P. and Müller-Eberhard, H.-J., J. Exp. Med. 143 : 1131-1139, 1976) and C9 (Podack, E.R., Tschopp, J. and Müller-Eberhard, H.-J., J. Exp. Med. 156 : 268-282) are purified in

20 accordance with standard methods. One μg of C5b-6 which lyses, in the absence of CLI, about 80% of the erythrocytes under the conditions set forth below, is mixed with 30 μl of sheep erythrocytes in 10 mmol veronal buffer, pH 7.4, 142 mmol NaCl, 0.1% gelatin

25 and 10 mmol EDTA (abbreviated GVBE), and incubated for 20 minutes at 25° C. Then C7 (1 $\mu\text{g}/\text{ml}$ final concentration), C8 (0.2 $\mu\text{g}/\text{ml}$ final concentration) and C9 (1 $\mu\text{g}/\text{ml}$ final concentration), as well as CLI in various concentrations in a range from 0.25 $\mu\text{g}/\text{ml}$ to 50 $\mu\text{g}/\text{ml}$

30 are added thereto in a volume of, in total, 30 μl .

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Prior to addition, the four proteins had been pre-incubated for 5 minutes at room temperature. After an incubation of 30 minutes at 37° C, the intact erythrocytes are removed by centrifuging (1500 rpm, 2 minutes), and the amount of hemoglobin released into the supernatant is determined at a wavelength of 412 nm in a spectrophotometer.

(b) Inhibition of Perforin-Mediated Lysis
by CLI

10 Perforin is purified according to the method
by Masson, D., and Tschopp, J., J. Biol. Chem. 260 :
9069-9072, 1985. First of all, 30 µl of sheet erythro-
cytes (1×10^9 cells/ml) is preincubated with 30 µl of
CLI (various concentrations) for 10 minutes at room
15 temperature. Perforin, which is diluted in GVBE to such
an extent that 80% of the erythrocytes is lysed during
the test, is admixed thereto in 30 µl of buffer volume.
After 30 minutes of incubation at 37° C, the extent of
erythrocyte lysis is determined, as described above in
20 connection with the complement lysis test.

(c) Inhibition of Staphylococcus-α-Hemolysin-
Mediated Lysis by CLI

Staphylococcus aureus is among the most
frequent bacterial pathogens in man. The α-hemolysin
25 formed by almost all human-pathogenic Staphylococcus
aureus strains is presently considered to be an
important pathogenicity factor. S. aureus secretes
α-hemolysin as a water-soluble monomeric protein with
a molecular weight of 34,000. On the plasma membrane of
30 eucaryotic target cells, respectively 6 toxin monomers
group together into a hydrophilic, transmembranous

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channel having a diameter of 1-2 mm. Thereby, the ionic environment in the interior of the cell is disturbed; a rapid influx of calcium occurs from the extracellular space, and, in the extreme case, a
5 colloid-osmotically caused lysis of the target cell takes place. In sublytic concentrations, the arachidonic acid cascade can be activated with release of highly active prostaglandins and leukotrienes in endothelial cells. Human blood platelets are
10 particularly sensitive with respect to subcytolytic doses of staphylococcus- α -hemolysin. Due to the action of the toxin on platelets, platelet aggregation occurs, along with reduction of the clotting time in the platelet-rich plasma (Bhakdi et al., J. Exp. Med.,
15 168 : 527-542, 1988).

Previously, it has been assumed that specific neutralizing antibodies in the blood plasma and β -lipoproteins (plasma LDL) represent the only factors capable of inactivating the staphylococcus- α -hemolysin
20 molecules.

In order to confirm the biological activity of CLI with respect to staphylococcus- α -hemolysin, freshly washed rabbit erythrocytes are utilized in the test system described below. Staphylococcus- α -
25 hemolysin (staphylolysin reagent, 5 units per bottle, Behringwerke, Marburg) is diluted in GVB buffer (GVBE buffer without EDTA, see above) and preincubated at 37° C for one-half hour. Thereafter, 50 μ l of a rabbit erythrocyte suspension (10^8 /ml) is admixed to 200 μ l
30 of this solution. After incubating for one-half hour at 37° C, the specimens are centrifuged (2 minutes at 12,000 g's), and the released hemoglobin in the supernatant is measured by spectrophotometry at 412 nm. Total hemolysis is measured after adding sodium dodecyl

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sulfate (final concentration, 0.2% by weight). A 1:400 dilution of a working solution with 0.04 unit/ml results in a 71% hemolysis. This toxin dilution (100 units/ml) is preincubated with various CLI concentrations for one-half hour at 37° C, then 50 μ l of the rabbit erythrocyte suspension is added, and, after another 30 minutes at 37° C, the hemolysis is measured as above in the various batches. Figure 8 shows the measuring results of these tests. Even below physiological concentration (normal range: 50 - 100 μ g/ml), at approximately 15 μ g CLI/ml, the activity of the staphylococcus- α -hemolysin is almost entirely suppressed.

The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

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Figure 1 shows a scheme of the recombinant pGEM4/CLI-1 plasmid containing the entire coding sequence for the cytolysis inhibitor, and several important restriction scission sites. The plasmid has an ampicillin resistance gene coding for the enzyme beta-lactamase, and includes the Sp6 and, respectively, T7 promoter in the direct vicinity to the cloning scission site BamHI in the polylinker. The two ends of the cDNA insertion are provided with the adapter oligomers A and B according to Haymerle, H., Herz., J., Bressan, G.M., Frank, R. and Stanley, K.K., Nucleic Acids Res. 154 : 8615-8624, 1986. This plasmid was deposited in E. coli K12 on March 28, 1989 with DSM under DSM No. 5269.

Figure 2 shows the nucleotide base sequence of the CLI-1 clone and the amino acid sequence derived therefrom in the line therebelow. The amino-terminal sequences of the a-chain (amino acids 1 to 205) and the b-chain (amino acids 206 to 427) are underlined. On the left-hand margin of the figure, the numbering is indicated for the nucleotide bases, and on the right-hand margin of the figure the numbering of the amino acids is set forth.

Figure 3 shows the results of the Southern analysis: the position of the molecular weight markers is indicated in kilobases on the left-hand margin of the figure.

Figure 4a shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (10% polyacrylamide proportion) under nonreducing (left) and reducing conditions (right).

Figure 4b shows the purified CLI after separation by SDS polyacrylamide gel electrophoresis (12% polyacrylamide proportion).

Figure 5: Western Blot identification (bars 1 to 3') of CLI in human plasma (1), in purified SC5b-9 complex (2), in seminal fluid (3 and 3'). Bar 4 shows the proteins of the seminal fluid after staining the SDS gel with "Commassie" blue.

Figure 6: Inhibition of complement lysis by CLI: CLI inhibits lysis of the erythrocytes by soluble C5b-6, C7, C8 and C9 in concentration-dependent way (bottom curve), but does not inhibit lysis of C5b-7 erythrocyte intermediate stages by C8 and C9 (top curve).

Figure 7: Inhibition of perforin-mediated erythrocyte lysis at various CLI concentrations.

Figure 8: Inhibition of staphylococcus- α -hemolysin-mediated erythrocyte lysis by CLI. Staphylococcus- α -hemolysin is preincubated with varying CLI concentrations at 37° C in GVB buffer and thereafter mixed with a rabbit erythrocyte suspension in GVB buffer. Subphysiological CLI concentrations (15 μ g/ml) almost completely suppress the toxin-caused lysis of the erythrocytes.

Figure 9: ApoA-I-CLI complexes appear in the electron microscope as spherical structures having a diameter of 9.0 ± 1 nm.

WHAT IS CLAIMED IS:

1. An isolated DNA sequence coding for a blood plasma component having a biological activity of inhibiting cytolysis mediated by a cytolytic protein.
2. A DNA sequence of claim 1, wherein the cytolytic protein is perforin, complement protein, or α -hemolysin.

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3. A DNA sequence of claim 1, corresponding to the sequence

CTGCGAACCCTCTCTACTCTCCGAAGGGAATTGTCCTTCCTGGCTTCCAC
TACTTCCACCCCTGAATGCACAGGCAGCCCGGCCCAAGTCTCCCACTA
GGGATGCAGATGGATTTCGGTGTGAAGGGCTGGCTGCTGTTGCCTCCGGCTCT
TGAAGTCAAGTTCAGAGGCGTGCAAAGACTCCAGAATTGGAGGCATG
ATGAAGACTCTGCTGCTGTTTGTGGGGCTGCTGCTGACCTGGGAGAGTGGGC
AGGTCTGGGGGACCAGACGGTCTCAGACAATGAGCTCCAGGAAATGT
CCAATCAGGGAAGTAAGTACGTCAATAAGGAAATTCAAAATGCTGTCAACGG
GGTGAAACAGATAAAGACTCTCATAGAAAAACAAACGAAGAGCGCAA
GACACTGCTCAGCAACCTAGAAGAAGCCAAGAAGAAGAAAGAGGATGCCCTA
AATGAGACCAGGGAATCAGAGACAAAGCTGAAGGAGCTCCAGGAGTG
TGCAATGAGACCATGATGGCCCTCTGGGAAGAGTGTAAGCCCTGCCTGAAAC
AGACCTGCATGAAGTTCTACGCACGCGTCTGCAGAAGTGGCTCAGGCC
TGGTTGGCCGCCAGCTTGAGGAGTTCCTGAACCAGAGCTCGCCCTTCTACTTC
TGGATGAATGGTGACCGCATCGACTCCCTGCTGGAGAACGACCGGCA
GCAGACGCACATGCTGGATGTCATGCAGGACCACTTCAGCCGCGCGTCCAGC
ATCATAGACGAGCTCTTCCAGGACAGGTTCTTCACCCGGGAGCCCCAG
GATACCTACCACTACCTGCCCTTCAGCCTGCCCCACCGGAGGCCTCACTTC
TTCTTTCCCAAGTCCCGCATCGTCCGAGCTTGATGCCCTTCTCTCCGT
ACGAGCCCCTGAACTTCCACGCCATGTTCCAGCCCTTCCTTGAGATGATAC
ACGAGGCTCAGCAGGCCATGGACATCCACTTCCACAGCCCGGCCTTCCA
GCACCCGCCAACAGAATTCATACGAGAAGGCGACGATGACCGGACTGTGTG
CCGGGAGATCCGCCACAACCTCCACGGGCTGCCTGCGGATGAAGGACCAG
TGTGACAAGTGCCGGGAGATCTTGTCTGTGGACTGTTCCACCAACAACCCC
TCCCAGGCTAAGCTGCGGCGGGAGCTCGACGAATCCCTCCAGGTCGCTG
AGAGGTTGACCAGGAAATATAACGAGCTGCTAAAGTCCTACCAGTGGAAGA
TGCTCAACACCTCCTCCTTGCTGGAGCAGCTGAACGAGCAGTTTAACTG

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GGTGTCCCGGCTGGCAAACCTCACGCAAGGCGAAGACCAGTACTATCTGCG
GGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTTCCCTCCGGT
GTCAGTGAAGTGGTCGTGAAGCTCTTTGACTCTGATCCCATCACTGTGACG
GTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTGG
CGGAGAAAGCGCTGCAGGAATACCGCAAAAAGCACCGGGAGGAGTGAGATG
TGGATGTTGCTTTTGCACCTACGGGGGCATCTGAGTCCAGCTCCCCCA
AGATGAGCTGCAGCCCCCAGAGAGAGCTCTGCACGTCACCAAGTAACCAGGC

4. A DNA sequence of claim 3 having an open reading frame beginning at the sequence corresponding to nucleotide 199.

5. A DNA sequence of claim 4, wherein, upon expression of said sequence in a host cell, a 21 amino acid hydrophobic signal peptide coded for by the first 63 bases of the open reading frame is split off from the remainder of the amino acid sequence during translocation into the endoplasmic reticulum.

6. A DNA sequence of claim 3, which is an allelic variant of the specified sequence.

7. A DNA sequence of claim 3, which is a sequence differing from the specified sequence by degenerate codons.

8. A vector comprising a DNA sequence of claim 1.

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9. A vector comprising a DNA sequence of claim 3.
10. Plasmid pGEM4/CLI-1, deposited in E. coli as DSM No. 5269, a vector of claim 8.
11. A vector plasmid of claim 8 which is expressible in eucaryotes.
12. A host organism, transformed with a vector plasmid of claim 8.
13. A host organism, transformed with the vector plasmid of claim 10.
14. E. coli, transformed with the vector plasmid of claim 10.
15. An isolated cytolysis inhibitor protein having the biological activity of inhibiting cell lysis mediated by a cytolytic protein.
16. A cytolysis inhibitor of claim 15, wherein the cell lysis is mediated by a complement protein, perforin, or α -hemoglobin.
17. A cytolysis inhibitor of claim 15, corresponding to the amino acid sequence

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M K T L L L F V G L L L T W E S G Q
 -21
 V L G D O T V S D N E L Q E M S 13
 N Q G S K Y V N K E I Q N A V N G 46
 V K Q I K T L I E K T N E E R K 79
 T L L S N L E E A K K K K E D A L 113
 N E T R E S E T K L K E L P G V 146
 C N E T M M A L W E E C K P C L K Q 179
 T C M K F Y A R V C R S G S G L 213
 V G R Q L E E F L N Q S S P F Y F 246
 W M N G D R I D S L L E N D R Q 279
 Q T H M L D V M Q D H F S R A S S 313
 I I D E L F Q D R F F T R E P Q 346
 D T Y H Y L P F S L P H R R P H F 379
 F F P K S R I V R S L M P F S P Y 413
 E P L N F H A M F Q P F L E M I H 427
 E A Q Q A M D I H F H S P A F Q
 H P P T E F I R E G D D D R T V C
 R E I R H N S T G C L R M K D Q
 C D K C R E I L S V D C S T N N P
 S Q A K L R R E L D E S L Q V A E
 R L T R K Y N E L L K S Y Q W K M
 L N T S S L L E Q L N E Q F N W
 V S R L A N L T Q G E D Q Y Y L R
 V T T V A S H T S D S D V P S G
 V T E V V V K L F D S D P I T V T
 V P V E V S R K N P K F M E T V A
 E K A L Q E Y R K K H R E E

18. A cytolysis inhibitor of claim 15, which is human cytolysis inhibitor.

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19. A cytolysis inhibitor of claim 17, which is glycosylated.

20. A bioactive polypeptide comprising a mutant or fragment of a cytolysis inhibitor of claim 15, which retains the biological activity of the cytolysis inhibitor.

21. A pharmaceutically acceptable salt of a cytolysis inhibitor of claim 15.

22. A pharmaceutical composition comprising a cytolysis inhibitor of claim 15 or a physiologically acceptable salt thereof and a pharmaceutically acceptable excipient.

23. An antibody which specifically binds to a portion of a cytolysis inhibitor protein of claim 15.

24. A monoclonal antibody which specifically binds to a portion of a cytolysis inhibitor protein of claim 15.

25. Monoclonal antibody CLI-9, a monoclonal antibody of claim 24.

26. A hybridoma cell line which produces a monoclonal antibody of claim 24.

27. Hybridoma cell line CLI-9, ECACC No. 89 05 26 02, a hybridoma cell line which produces the monoclonal antibody of claim 25.

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28. A process for producing an isolated cytolysis inhibitor protein having the biological activity of inhibiting cytolysis mediated by a cytolytic protein, comprising expressing a recombinant vector containing a DNA sequence coding for said cytolysis inhibitor protein in a host cell.

29. An isolated recombinant cytolysis inhibitor produced by a process of claim 28.

30. A process for producing an isolated natural cytolysis inhibitor protein having the biological activity of inhibiting cytolysis mediated by a cytolytic protein, comprising subjecting a bodily fluid containing said cytolysis inhibitor protein to affinity chromatography over a solid support to which is attached an affinity agent specific for the cytolysis inhibitor, wherein the affinity agent is a monoclonal antibody of claim 24.

31. A method of treating a condition mediated by cytolytic protein-mediated cell lysis, comprising administering an anti-cytolytically-effective amount of a cytolysis inhibitor of claim 15.

32. A method of claim 31, wherein the condition is inflammation, immunological disorder, infertility, tissue destruction mediated by complement- or perforin-induced cell lysis, or is caused by a membrane-active cytolytic toxin, peptide, and/or protein produced by a pathogenic organism, fungus, or insect.

33. A method of claim 31, wherein the condition is caused by a congenital or acquired deficit of endogenous cytolysis inhibitor or natural CLI plasma component.

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34. A method of inducing a contraceptive effect, comprising administering a vaccine which comprises an amount of an immunogenically effective portion of a cytotoxic inhibitor protein of claim 15 and/or the natural carbohydrate portion of said protein, effective to induce an immunogenic response to endogenous cytotoxic inhibitor, and thereby effective to induce a contraceptive effect.

35. In a method of detecting and optionally quantitatively determining the presence of a substance in bodily fluids using an immunological molecule, the improvement wherein the substance to be detected and optionally quantitatively determined is a cytotoxic inhibitor or a cytotoxic inhibitor-containing complex and the immunological molecule is an antibody of claim 23.

36. A method of claim 35, which is a method of diagnosis of a condition or disease wherein changes occur in the amount of cytotoxic inhibitor or wherein a cytotoxic inhibitor reaction product is produced which retains immunospecificity to said antibody.

37. An isolated CLI-ApoA-I or CLI-HDL complex comprising an isolated natural or recombinant cytotoxic inhibitor complexed with ApoA-I, with or without synthetic or natural lipids.

38. A method of treating a condition caused by a disturbance in lipoprotein metabolism or a degenerative vascular condition, comprising administering an effective amount of a cytotoxic inhibitor or a CLI-ApoA-I complex or CLI-HDL complex of claim 37.

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39. A method of claim 38, wherein the condition is arteriosclerosis.

40. A method of claim 35, wherein the substance is a cytolysis inhibitor-containing complex comprising CLI-ApoA-I or CLI-HDL, further comprising ApoA-I-specific antibodies.

41. In a method of immunohistological differential diagnosis or in vivo diagnosis involving the determination of the presence and/or location of a substance in bodily tissues using an immunological molecule, the improvement wherein the substance to be determined and/or located is a cytolysis inhibitor or a cytolysis inhibitor-containing complexes and the immunological molecule is a monoclonal antibody of claim 24.

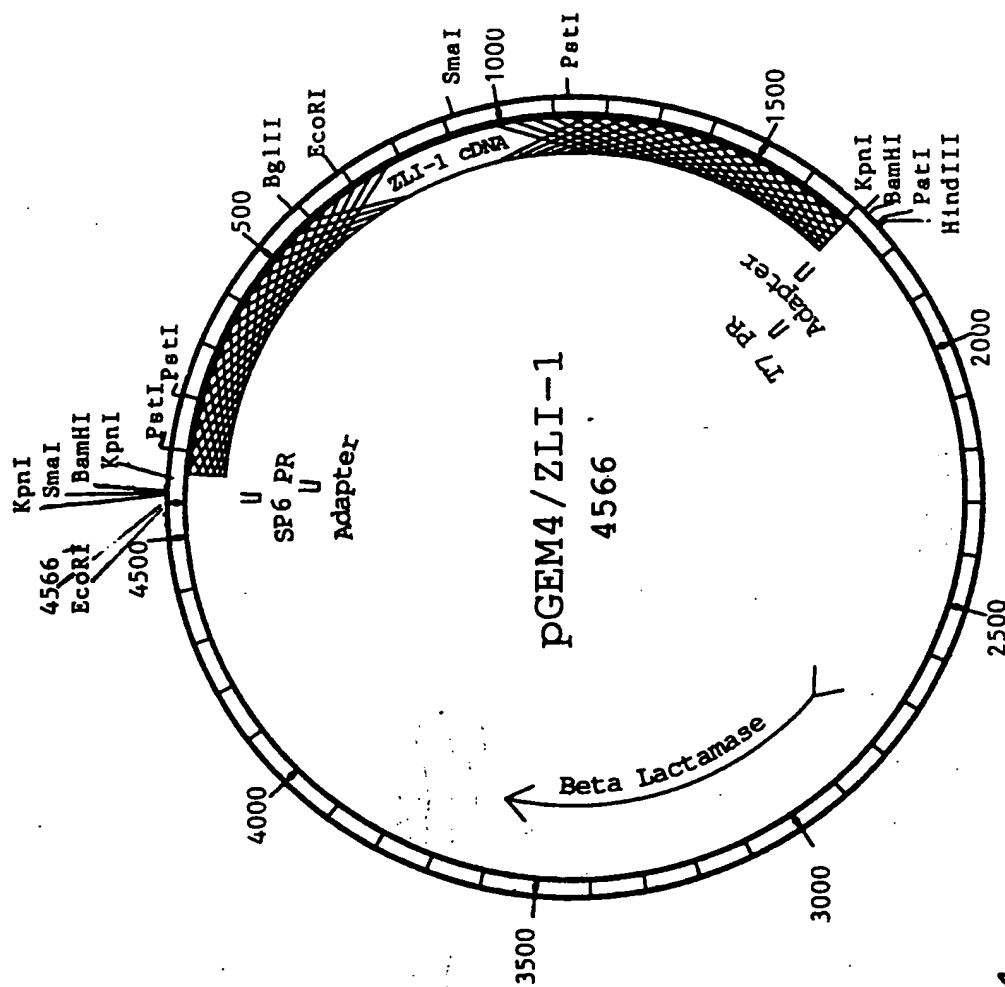


Fig. 1

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1 CTGCGAACCCTCTCTACTCTCCGAAGGGAATTGTCCTTCCTGGCTTCCAC
TACTTCCACCCCTGAATGCACAGGCAGCCCGGCCCAAGTCTCCCACTA
99 GGGATGCAGATGGATTTCGGTGTGAAGGGCTGGCTGCTGTTGCCTCCGGCTCT
TGAAAGTCAAGTTCAGAGGCGTGCAAAGACTCCAGAATTGGAGGCATG
199 ATGAAGACTCTGCTGCTGTTTGTGGGGCTGCTGCTGACCTGGGAGAGTGGGC
M K T L L L F V G L L L T W E S G Q
-21
AGGTCCTGGGGGACCAGACGGTCTCAGACAATGAGCTCCAGGAAATGT
V L G D O T V S D N E L Q E M S 13
-1 +1
299 CCAATCAGGGAAGTAAGTACGTCAATAAGGAAATTCAAAATGCTGTCAACGG
N Q G S K Y V N K E I Q N A V N G
GGTGAAACAGATAAAGACTCTCATAGAAAAACAAACGAAGAGCGCAA
V K Q I K T L I E K T N E E R K 46
399 GACACTGCTCAGCAACCTAGAAGAAGCCAAGAAGAAGAAAGAGGATGCCCTA
T L L S N L E E A K K K K E D A L
AATGAGACCAGGGAATCAGAGACAAAGCTGAAGGAGCTCCAGGAGTG
N E T R E S E T K L K E L P G V 79
499 TGCAATGAGACCATGATGGCCCTCTGGAAGAGTGTAAGCCCTGCCTGAAAC
C N E T M M A L W E E C K P C L K Q
AGACCTGCATGAAGTTCTACGCACGCGTCTGCAGAAGTGGCTCAGGCC
T C M K F Y A R V C R S G S G L 113
599 TGGTTGGCCGCCAGCTTGAGGAGTTCCTGAACCAGAGCTCGCCCTTCTACTTC
V G R Q L E E F L N Q S S P F Y F
TGGATGAATGGTGACCGCATCGACTCCCTGCTGGAGAACGACCGGCA
W M N G D R I D S L L E N D R Q 146
699 GCAGACGCACATGCTGGATGTCATGCAGGACCACTTCAGCCGCGCGTCCAGC
Q T H M L D V M Q D H F S R A S S
ATCATAGACGAGCTCTTCCAGGACAGGTTCTTCACCCGGGAGCCCCAG
I I D E L F Q D R F F T R E P Q 179
799 GATACCTACCACTACCTGCCCTTCAGCCTGCCCCACCGGAGGCCTCACTTC
D T Y H Y L P F S L P H R R P H F
TTCTTTCCCAAGTCCCGCATCGTCCGCAGCTTGATGCCCTTCTCTCCGT
F F P K S R I V R S L M P F S P Y 213
899 ACGAGCCCCTGAACTTCCACGCCATGTTCCAGCCCTTCCTTGAGATGATAC
E P L N F H A M F Q P F L E M I H
ACGAGGCTCAGCAGGCCATGGACATCCACTTCCACAGCCCGGCCTTCCA
E A Q Q A M D I H F H S P A F Q 246
999 GCACCCGCCAACAGAATTCATACGAGAAGGCGACGATGACCGGACTGTGTG
H P P T E F I R E G D D D R T V C

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CCGGGAGATCCGCCACAACCTCCACGGGCTGCCTGCGGATGAAGGACCAG
R E I R H N S T G C L R M K D Q 279

1099 TGTGACAAGTGCCGGGAGATCTTGTCTGTGGACTGTTCCACCAACAACCCC
C D K C R E I L S V D C S T N N P

TCCCAGGCTAAGCTGCGGCGGGAGCTCGACGAATCCCTCCAGGTCGCTG
S Q A K L R R E L D E S L Q V A E 313

1199 AGAGGTTGACCAGGAAATATAACGAGCTGCTAAAGTCCTACCAGTGGAAGA
R L T R K Y N E L L K S Y Q W K M

TGCTCAACACCTCCTCCTTGCTGGAGCAGCTGAACGAGCAGTTTAACTG
L N T S S L L E Q L N E Q F N W 346

1299 GGTGTCCCGGCTGGCAAACCTCAGCAAGGCGAAGACCAGTACTATCTGCG
V S R L A N L T Q G E D Q Y Y L R

GGTCACCACGGTGGCTTCCCACACTTCTGACTCGGACGTTTCTTCCGGT
V T T V A S H T S D S D V P S G 379

1399 GTCACTGAGGTGGTCGTGAAGCTCTTTGACTCTGATCCCATCACTGTGACG
V T E V V V K L F D S D P I T V T

GTCCCTGTAGAAGTCTCCAGGAAGAACCCTAAATTTATGGAGACCGTGG
V P V E V S R K N P K F M E T V A 413

1499 CGGAGAAAGCGCTGCAGGAATACCGCAAAAAGCACCGGGAGGAGTGAGATG
E K A L Q E Y R K K H R E E 427

TGGATGTTGCTTTTGCACCTACGGGGGCATCTGAGTCCAGCTCCCCCA

1599 AGATGAGCTGCAGCCCCCAGAGAGAGCTCTGCACGTCACCAAGTAACCAGGC 1651

Fig. 2

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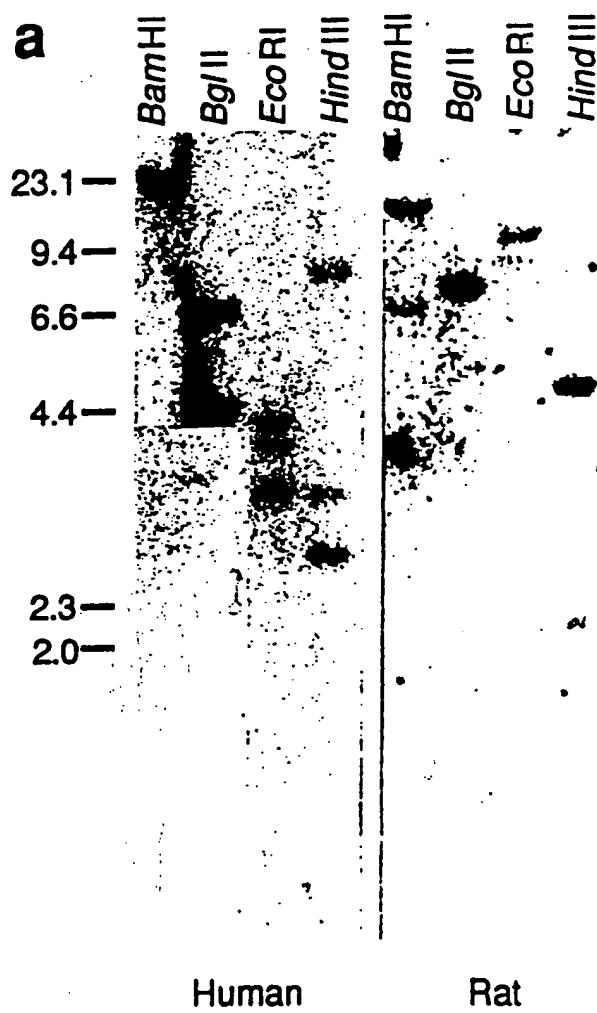


Fig. 3

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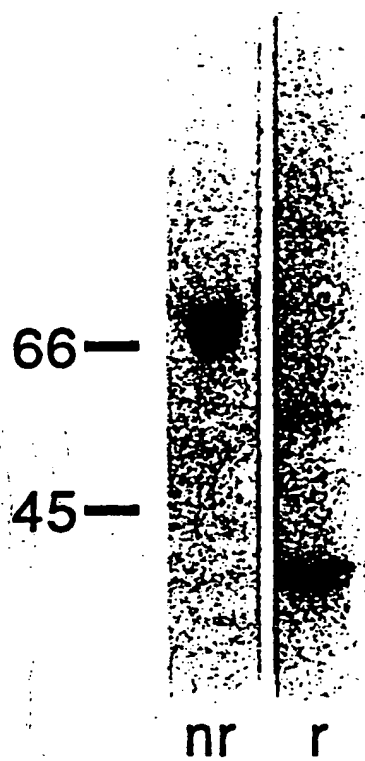
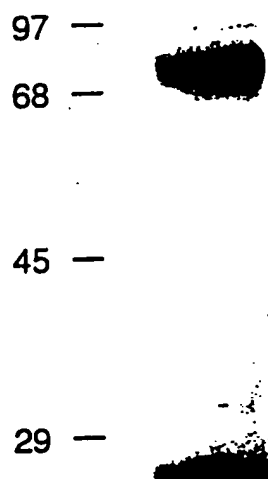


Fig. 4

Fig. 4b



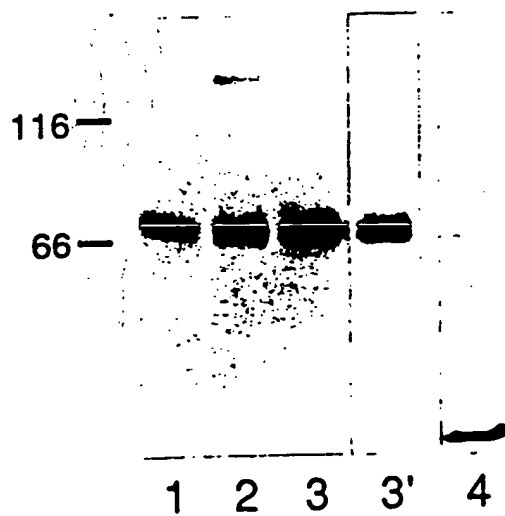


Fig. 5

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INHIBITION OF COMPLEMENT CYTOLYSIS

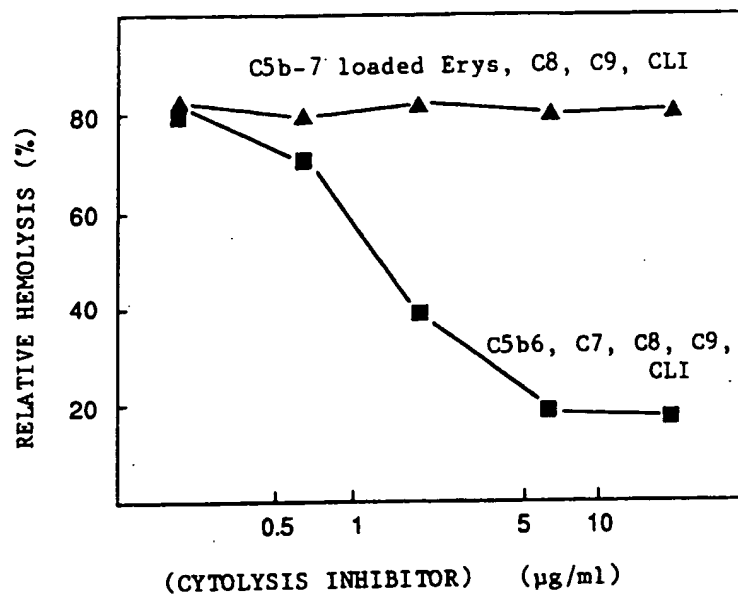


Fig. 6

INHIBITION OF CTL/NK PERFORIN CYTOLYSIS

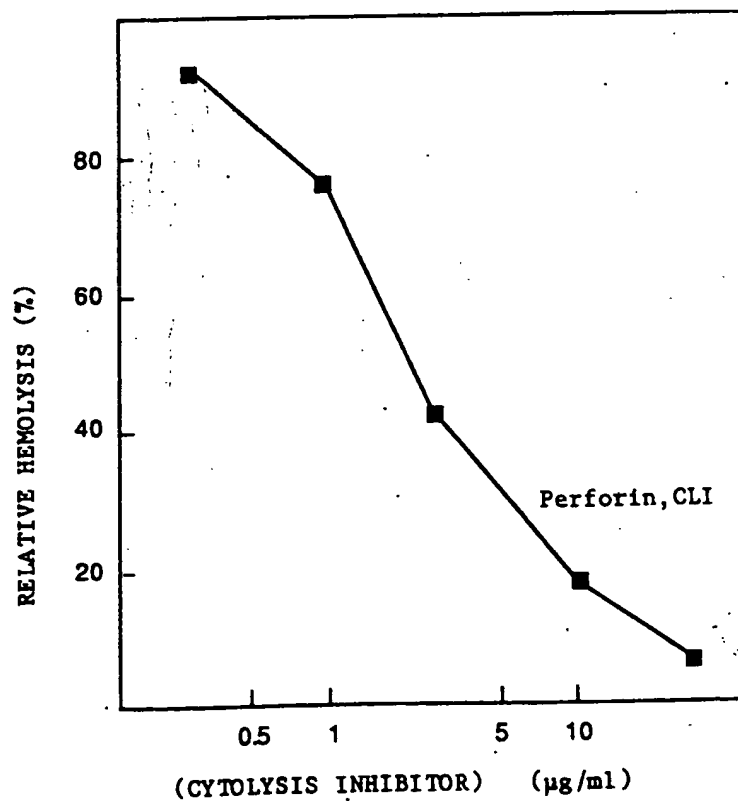


Fig. 7

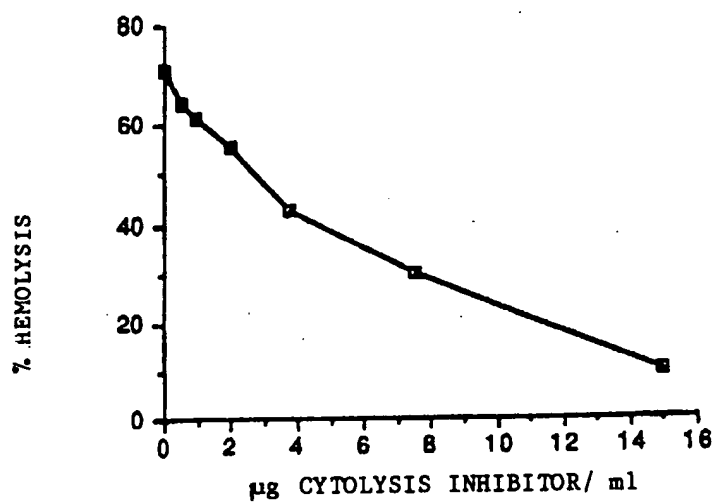
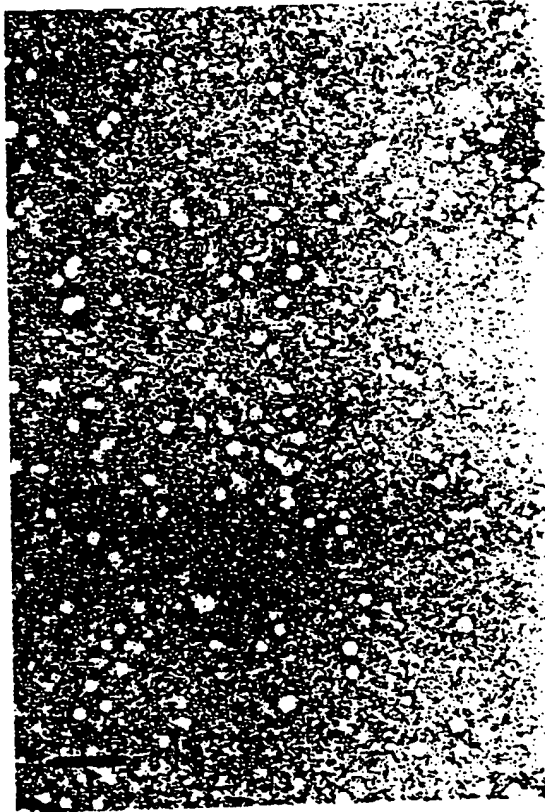
*Fig. 8***SUBSTITUTE SHEET**


Fig. 9



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INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/01677

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ C 12 N 15/12, 1/21, C 12 P 21/02, A 61 K 37/02, IPC ⁵ : C 12 P 21/08, G 01 N 33/68, C 07 K 13/00		
II. FIELDS SEARCHED Minimum Documentation Searched ?		
Classification System	Classification Symbols	
IPC ⁵	C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	Proc. Natl. Acad. Sci. USA, volume 86, September 1989, (Washington, DC; US), D.E. Jenne et al.: "Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: Identity to sulfated glycoprotein 2, a constituent of rat testis fluid", pages 7123-7127 see the whole article --	1-30,35
X	The EMBO Journal, volume 8, no. 3, March 1989, IRL Press, (Oxford, GB), L. Kirszbaum et al.: "Molecular cloning and characterization of the novel, human complement-associated protein, SP-40,40: a link between the complement and reproductive systems", pages 711-718 see the whole article --	1-30,35
./.		
* Special categories of cited documents: ** "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art. "A" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 10th December 1990	Date of Mailing of this International Search Report 22. 01. 91	
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer  Natalie Weinberg	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,X

Biochemistry, volume 29, no. 22, 1990,
American Chemical Society,
H.V. de Silva et al.: "Apolipoprotein
J: Structure and tissue distribution",
pages 5380-5389
see the whole article

1-30,35,37

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers **, because they relate to subject matter not required to be searched by this Authority, namely:

** Unsearchable Claims 31-34, 38,39

Partly unsearchable claims 41 (only as far as it concerns no invivo
diagnosis)

See Rule 39.1 (iv): methods for treatment of the human or animal body
by surgery or therapy, as well as diagnostic methods,

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers _____, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This international Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.